Title: Notch Signaling in Acquired Middle Ear Cholesteatoma

Abstract

Hypothesis: We hypothesized that an anomalous change of Notch signaling might be involved in the pathophysiology of cholesteatoma.

Background: The Notch signaling pathway regulates integrated growth and differentiation control of keratinocytes. Its involvement in cholesteatoma proliferation has not been elucidated.

Methods: We obtained cholesteatoma and external auditory canal (EAC) skin samples from patients with middle ear cholesteatoma who underwent tympanomastoid surgery. We performed polymerase chain reaction using the RT^2 Profiler™ PCR Array Human Notch Signaling Pathway (Qiagen) in the cholesteatoma and EAC skin samples (n = 6 each). This was followed by immunohistochemical staining of Notch1, enhancer of split-1 (HES1), and p53 in 41 and 8 cholesteatoma and EAC skin samples, respectively.

Results: The fold change of *Notch1* gene expression was lowest in cholesteatoma, with a statistically significant difference ($p = 0.0424$). Moreover, the fold change of *HES1* expression decreased ($p = 0.272$). The positive rates of Notch1 and HES1 protein expressions in the cholesteatoma (48.5 ± 32.4% and 44.9 ± 17.8%, respectively) were significantly lower than in the EAC skin (83.4 ± 17.5% and 55.7 ± 7.1%, respectively) ($p < 0.001$ and $p < 0.01$). In contrast, the positive rate of p53 expression in the cholesteatoma (8.5 ± 11.4%) was significantly higher than in the EAC skin (0.5 ± 0.7%) ($p < 0.001$).

Conclusion: The decreases in Notch1 and HES1 protein expression might play an important role in the hyperproliferative character of the keratinizing squamous epithelium in cholesteatoma. An increase in p53

might reflect the reaction to cellular hyperproliferation.

Keywords: Cholesteatoma, Notch signaling, Notch1, HES1, p53

INTRODUCTION

Acquired middle ear cholesteatoma is a non-neoplastic lesion in the temporal bone that arises from an abnormal growth of the keratinizing squamous epithelium from the external auditory canal (EAC) to the tympanic membrane[1]. It gradually expands and results in bone erosion of the nearby structures, thus causing complications[2]. Despite substantial research into the disorder, the etiopathogenesis of acquired cholesteatoma has not been clearly elucidated. Moreover, no viable nonsurgical therapy has been developed so far. Cholesteatoma is not a tumor; rather, it is clinically characterized by its resemblance to a neoplasm. It is reportedly considered an example of uncontrolled cell growth, capable of altering the balance toward cellular hyperproliferation and enhancing the capacity for invasion and osteolysis[3]. The dysregulation of cell growth control involves internal genomic or epigenetic alterations and external stimuli, including an involvement of extracellular and intracellular signal transduction cascades[3].

Notch is a transmembrane receptor that is expressed on the cell membrane. The information transmitted through Notch is called Notch signaling, which is a highly conserved molecular network. Researchers have identified the expression of four receptor homologs (Notch1–4) and five ligand homologs (Jagged [Jag] 1, Jag2, Delta-like (Dll) 1, Dll3, Dll4), which are transmembrane molecules, on various cells in mice and humans[4]. Once activated by a ligand, the intracellular domain of the Notch receptor (NICD) is cleaved by γ-secretase, thus leading to the translocation of the NICD into the nucleus. NICD associates with the transcription factor CSL (*C*entromere-binding factor 1/*S*uppressor of Hairless/*L*ag-1)[5] to generate a transactivation complex that initiates the transcription of target genes, such as the hairy and enhancer of

split-1 (*HES1*) transcriptional repressor[6]. The function of Notch signaling is varied and diverse across cells. While it promotes tissue growth and cancers in some circumstances, it leads to cell death and tumor suppression in others[7]. The Notch pathway in the skin is involved in the integrated growth/differentiation control of keratinocytes and the maintenance of normal skin homeostasis[8].

TP53 is a well-known tumor suppressor gene whose loss increases cell proliferation and the risk of cancer[9]. *Notch1* has been shown to be a downstream positive target of p53 protein, which is a *TP53* gene product, in both normal and cancer-derived keratinocytes[10]. Transcriptional suppression of p53 reportedly leads to the negative regulation of *Notch1*, causing proliferation of keratinocytes and skin cancer[11].

We hypothesized that an anomalous change of Notch signaling might regulate the pathophysiology of hyperproliferation of the keratinizing squamous epithelium in cholesteatoma. To the best of our knowledge, no study has investigated the involvement of Notch signaling in cholesteatoma. We aimed to comprehensively assess gene expression related to Notch signaling in cholesteatoma epithelial tissue using a polymerase chain reaction (PCR) array. In addition, we aimed to conduct an immunohistochemical study to confirm the protein expression related to Notch signaling in the aforementioned tissue. Furthermore, we intended to assess the expression of p53 that reportedly controls the upstream expression of Notch1 in the Notch signaling pathway in normal and cancer-derived keratinocytes[10].

MATERIALS AND METHODS

Sample collection

We retrospectively evaluated patients with primary acquired middle ear cholesteatoma who underwent tympanomastoid surgery between February 2010 and April 2020 at our hospital. We excluded those with congenital and recurrent cholesteatoma. All patients provided written informed consent for participation. Our study was approved by the Institutional Review Board for clinical research of our university hospital and was conducted in accordance with the tenets of the Declaration of Helsinki. Cholesteatoma tissues were collected during tympanomastoid surgery. In contrast, the control tissues were collected from the EAC skin during the above-mentioned surgery. Both cholesteatoma and EAC skin tissues were collected from the same patients with middle ear cholesteatoma. Since cholesteatoma is characterized by a benign keratinizing squamous epithelial lesion, we adopted the EAC skin for the control, similarly to previous studies on cholesteatoma[12–14]. These samples were immediately fixed in 10% buffered formalin and embedded in paraffin for histopathological and immunohistochemical analyses. Some of the samples were immediately fixed with the PAXgene Tissue System (PreAnalytiX, Hombrechtikon, Switzerland) and embedded in paraffin, based on the manufacturer's protocol for PCR.

PCR array for gene expression related to Notch signaling

The PAXgene Tissue-fixed, paraffin embedded (PFPE) tissues were cut into 10-μm-thick sections with a microtome. Hematoxylin and eosin (HE) staining was performed to confirm adequate sample collection. A microdissection technique was used to collect the epithelial layer from the cholesteatoma and EAC skin samples. Total ribonucleic acid (RNA) was extracted and reverse transcribed using the PAXgene Tissue RNA Kit (PreAnalytiX) and RT^2 First Strand Kit (Qiagen, Maryland, USA), respectively, according to the

manufacturer's protocol. The cDNA was mixed with RT^2 SYBR Green ROX qPCR MasterMix (Qiagen). This mixture was added to a 96-well RT^2 mRNA PCR Array (RT^2 Profiler™ PCR Array Human Notch Signaling Pathway, Qiagen) that comprised primers for 84 relevant genes (and five housekeeping genes for reference) according to the manufacturer's instructions. Real-time PCR was performed with a StepOnePlus™ real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Values of the cycle threshold (Ct) obtained during quantification were used for calculating fold changes in the mRNA abundance using the $2^{-\Delta\Delta Ct}$ method. Two housekeeping genes, namely Glyceraldehyde-3-Phosphate Dehydrogenase and Ribosomal protein lateral stalk subunit P0, were used to normalize the transcript levels, following which the ΔCt was calculated for each gene of interest in the plate. A fold change ($2^{-\Delta\Delta Ct}$ method) was measured as the normalized gene expression ($2^{-\Delta Ct}$) in the test sample divided by that ($2^{-\Delta Ct}$) in the control sample.

Immunohistochemistry

Formalin-fixed paraffin embedded (FFPE) tissues were cut at a thickness of 4 μm. They were then deparaffinized in xylene, dehydrated through graded alcohols, and placed in 0.1% hydrogen peroxide to quench any endogenous peroxidase activity. Antigen retrieval was performed using a 750 W microwave oven for 15 min in 10 mM sodium citrate buffer (10 mmol/L, pH 6.0). The sections were blocked with 10% normal goat serum for 30 min at room temperature to prevent non-specific antibody binding. The slides were then incubated with an anti-Notch1 rabbit monoclonal antibody (#3608; Cell Signaling Technology Inc., Danvers, MA, USA) and anti-HES1 rabbit monoclonal antibody (#11988; Cell Signaling Technology Inc.) in a humid chamber at 4 °C overnight, and with an anti-p53 mouse monoclonal antibody (M7001; Dako,

Glostrup, Denmark) for 30 min at room temperature. This was followed by an incubation of the sections with horseradish peroxidase-labeled goat anti-rabbit or anti-mouse secondary antibody (Histofine MAX-PO (MULTI) kit; Nichirei, Tokyo, Japan) for 30 min at room temperature. The reaction products were observed by immersing the slides in freshly prepared diaminobenzidine solution for 10 min and counterstaining them with hematoxylin before dehydration and mounting.

Evaluation of immunostaining

The cytomembrane staining for Notch1 and the nuclear staining for HES1 and p53 were defined as antigen-positive cells. Positive cells throughout the entire keratinocyte layer were counted in three different areas at 400× magnification under a light microscope, and the average of the three percentages was calculated. In order to count as well as normal skin, we analyzed the epithelial layer only, rather than the entire cholesteatoma including the perimatrix. The staining intensity was not considered. Two independent investigators blinded to the clinical information examined the slides. We calculated the average of the two values and defined it as the percentage for the sample.

Statistical analyses

We conducted Welch's *t* test to analyze the differences in the ΔCt values for each gene and the antigen-positive cell percentages between the cholesteatoma and control group. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using JMP Pro 14 (SAS Institute, Inc., Cary, NC).

RESULTS

Expression of Notch signaling pathway-associated genes in cholesteatoma

The cholesteatoma samples for PFPE were obtained from five males and one female with a median age of 38.5 years (range 18–79 years). As controls, six EAC skin samples were collected from similar patients. HE staining showed that the samples had been collected adequately. We extracted the mRNA from these samples and performed a PCR array. Table 1 summarizes the fold change of each gene and the *p* value in the PCR array. The genes are arranged in an ascending order of their fold change. The names and descriptions of the genes are based on the Human Genome Organisation Gene Nomenclature Committee database. We could analyze 48 of the 84 gene expressions. More than half of the Ct values were missing, thus preventing an analysis of the remaining 36 genes. Figure 1 outlines a heatmap and the fold changes of gene expression. The fold change of *Notch1* expression in the cholesteatoma was the lowest with a statistically significant difference ($p = 0.042$). The fold change of *HES1* expression, one of the main targets of Notch signaling, had also decreased ($p = 0.272$). In contrast, the fold change of *Notch3* expression had increased ($p = 0.680$).

Expression of Notch1, HES1, and p53 in the immunohistochemical findings

The cholesteatoma samples for FFPE were obtained from 28 and 13 males and females, respectively, with a median age of 57 years (range 18–87 years). The control group consisted of eight EAC skin samples collected from other patients who underwent the surgery. It comprised four males and females each, with a median age of 53 years (range 13–82 years). These FFPE tissue samples were used in the immunohistochemical study. Notch1 expression was predominantly observed in the basal and suprabasal

layers of the cholesteatoma and normal EAC skin epithelium (Fig. 2). However, it expanded widely to the suprabasal layers in some of the normal EAC skin epithelium. The positive rate of Notch1 expression was 48.5 ± 32.4% and 83.4 ± 17.5% in the cholesteatoma and normal EAC skin epithelium, respectively (Fig. 3). A statistically significant difference was found in Notch1 expression between the cholesteatoma epithelium and the control ($p < 0.001$). The expression of HES1 in the cholesteatoma and the normal EAC skin epithelium was localized to the suprabasal layers (Fig. 2). The positive rate of HES1 expression was 44.9 ± 17.8% and 55.7 ± 7.1% in the cholesteatoma and normal EAC skin epithelium, respectively. Moreover, this difference was statistically significant ($p < 0.01$) (Fig. 3). The expression of p53 in the cholesteatoma and the normal EAC skin epithelium was predominant in the basal and suprabasal layers (Fig. 2). While the positive rate of p53 expression was 8.5 ± 11.4% in the cholesteatoma epithelium, it was 0.5 ± 0.7% in the normal EAC skin epithelium with a statistically significant difference ($p < 0.001$) (Fig. 3).

DISCUSSION

This is the first study that sheds light on the likely involvement of Notch1 in the pathophysiology of acquired middle ear cholesteatoma. The expression of *Notch1* mRNA in cholesteatoma tissues significantly decreased compared to that in normal EAC skin tissue. Immunohistochemical findings revealed that the primary expression sites of Notch1 and HES1 in the cholesteatoma epithelium were similar to those in the normal EAC skin epithelium. Nonetheless, their expressions in the cholesteatoma epithelium were significantly lower than that in the EAC skin epithelium. Figure 4 depicts the human Notch signaling pathway, based on

156 the Kyoto Encyclopedia of Genes and Genomes pathway database[15]. A solid box indicates the protein coded
157 by the genes analyzed in the PCR array. A dotted box indicates the protein coded by the genes that could not
158 be analyzed. A colored box indicates the coding genes regulated with a fold change of over two or less than
159 one-half, with pink and green colors indicating up- and down-regulation, respectively. The down-regulation
160 of the Notch1 receptor decreased the NICD that translocated into the nucleus, thereby reducing the
161 transactivation complex formed by an interaction with the transcription factor CSL. Moreover, it decreased
162 *HES1* mRNA, the target transcriptional product. Furthermore, we observed a decrease in the mRNA levels
163 of the Delta-like and Jagged ligands of Notch1 and that of NCSTN, which is a constituting factor of the γ-
164 secretase complex[16]. In contrast, the mRNA level of *Notch3* had increased. The Notch3 intracellular domain
165 (IC) is a poor activator, compared to Notch1 IC. It acts as a repressor by blocking the ability of the Notch1
166 IC to activate an expression through the *HES1* promoters[17,18]. In other words, an increased Notch3 exerts an
167 inhibitory influence on HES1 expression. Thus, the expression of the Notch1–HES1 signaling pathway
168 tended to decrease in cholesteatoma. Notch signaling reportedly leads to keratinocyte growth arrest and
169 differentiation[19]. Moreover, it plays a crucial role in determining the spinous cell fate from the basal cells[20].
170 Expression of *HES1* in keratinocytes leads to the downstream induction of the spinous layer genes encoding
171 differentiation-specific proteins[20]. A deletion of the *Notch1* gene in mouse skin resulted in epidermal and
172 corneal hyperplasia, followed by the development of skin tumors. It also facilitated chemical-induced skin
173 carcinogenesis, thus indicating *Notch1* functions as a tumor-suppressor gene[21]. The decreased expression of
174 Notch1 and HES1 in the cholesteatoma epithelium might alter the balance from cellular differentiation to

hyperproliferation and subsequently contribute to the pathological condition.

The immunohistochemical findings demonstrated that the expression of p53 in the cholesteatoma epithelium was significantly higher than that in the EAC skin epithelium. Some researchers have reported increased expression of p53 in the acquired cholesteatoma epithelium, compared to the controls[22–25]. The p53 protein suppresses cell proliferation by regulating the cell cycle arrest or by inducing apoptosis or both[23]. Cell proliferation could elevate their expression. In addition, neutrophils can also activate the p53 protein by releasing reactive oxygen species[24]. p53 expression in cholesteatoma could increase in response to cellular hyperproliferation and reactive oxygen species. However, there was no increase in *Notch1* that is reportedly a direct downstream target of p53[26]. In contrast, it decreased, notwithstanding the increased expression of p53. The increase in p53 and decrease in Notch1 protein in the cholesteatoma tissues seemed paradoxical. This can be explained by hypothesizing that the initial decrease of Notch1 protein expression and cell proliferation occurred because of some factors. This was followed by an increase in the p53 protein as a reaction to cell proliferation, mediated by a negative feedback control. However, some negative comments about increased p53 expression in the cholesteatoma epithelium [13,27] necessitate further research.

Our study had some limitations. First, the missing Ct values of some genes prevented the analysis of their expression. This can be attributed to the relatively small tissue sample size for PCR. Second, the small sample size might have reduced its statistical power. Third, mechanisms to induce the down-regulation of Notch1 in the cholesteatoma are still unknown, thereby necessitating further investigations.

CONCLUSION

This is the first study to suggest the possible involvement of Notch signaling in the pathophysiology of cholesteatoma. The decrease in Notch1 and HES1 protein expression might play an important role in hyperproliferation of the keratinizing squamous epithelium in cholesteatoma. The increase in p53 protein might be a reaction to cell proliferation, mediated by a negative feedback control. Further investigations are needed to reveal the relationship between Notch signaling and cholesteatoma.

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FIGURE LEGENDS

Fig. 1. A heatmap and the fold change of gene expression related to Notch signaling in the cholesteatoma (chole) and external auditory canal (EAC) skin. A gray-colored box indicates a missing value in the heatmap.

Fig. 2. Immunohistochemical staining of Notch1 (A, D), HES1 (B, E), and p53 (C, F) expression and localization in the cholesteatoma (A, B, and C) and external auditory canal (EAC) skin (D, E, and F). Original magnification: ×200.

Fig. 3. The expression of Notch1, HES1, and p53 in the cholesteatoma and external auditory canal (EAC) skin. The differences are analyzed using Welch's *t* test.

Fig. 4. The human Notch signaling pathway based on the Kyoto Encyclopedia of Genes and Genomes pathway database. A solid box indicates the protein coded by the genes we could analyze in the polymerase chain reaction array. A dotted box indicates the protein coded by the genes we could not analyze because of the missing cycle threshold values. A colored box indicates the coding genes regulated with a fold change of over two or less than one-half: A pink- and green-colored box indicates up- and down-regulation, respectively. The proteins and their corresponding genes are as follows: Delta-like: *DLL1* and *DLL4*; Jagged: *JAG1* and *JAG2*; Fringe: *LFNG* and *RFNG*; Notch: *NOTCH1*, *NOTCH2*, and *NOTCH3*; Deltex: *DTX1*; PSE2: *PSENEN*; PSEN: *PSEN1*; NCSTN: *NCSTN*; CSL: *RBPJL* and *RBPJ*; Hes1/5: *HES1* and *HES5*; SMRT:

277 *NCOR2*; and HDAC: *HDAC1* and *HDAC2*.

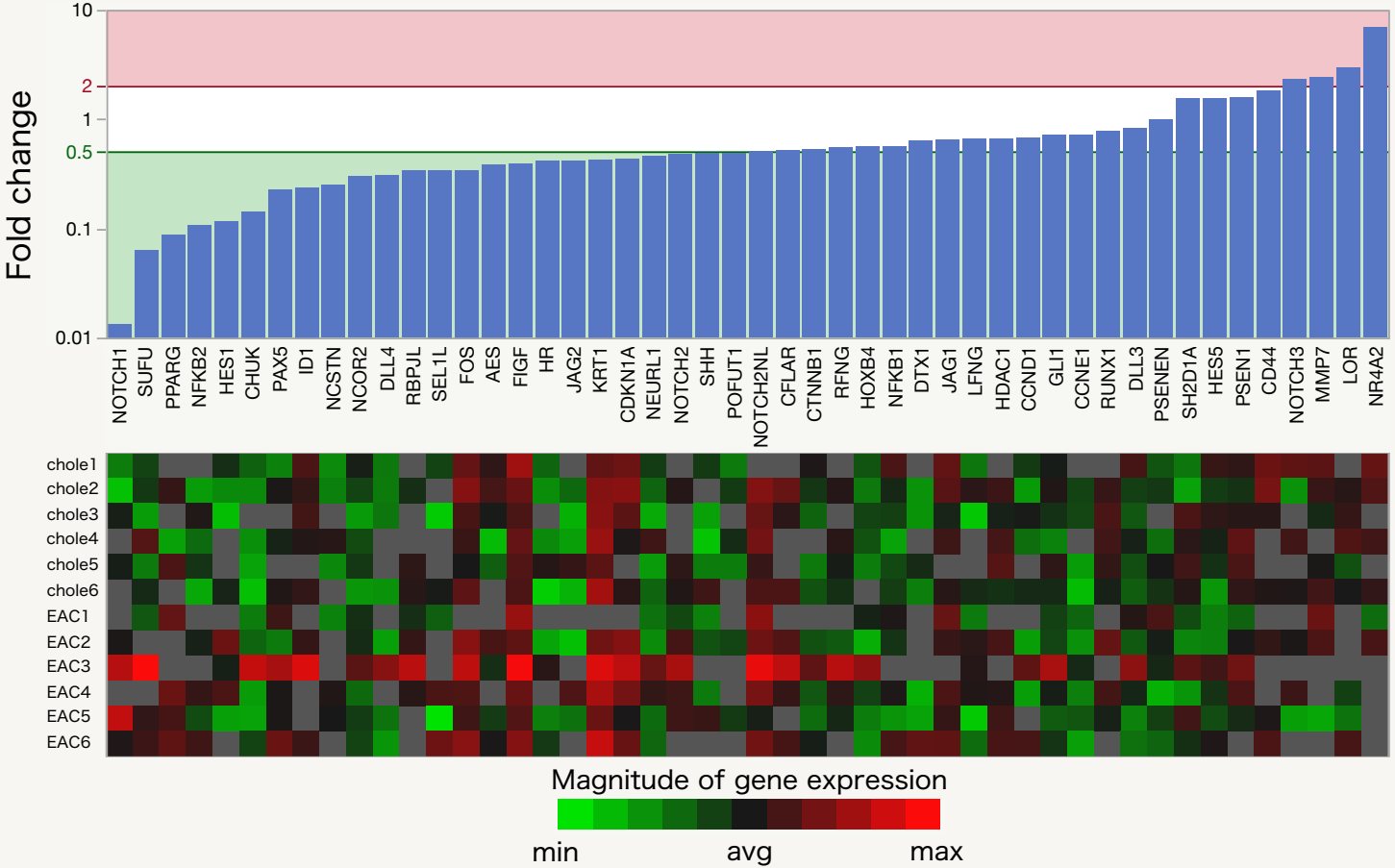
TABLE 1. Expression of the genes associated with the Notch signaling pathway in cholesteatoma

Gene symbol	Description	Fold change	*p* value
NOTCH1	Notch receptor 1	0.013	0.042
SUFU	SUFU negative regulator of hedgehog signaling	0.063	0.197
PPARG	Peroxisome proliferator activated receptor gamma	0.087	0.288
NFKB2	Nuclear factor kappa B subunit 2	0.108	0.072
HES1	Hes family bHLH transcription factor 1	0.117	0.272
CHUK	Component of inhibitor of nuclear factor kappa B kinase complex	0.142	0.209
PAX5	Paired box 5	0.226	0.253
ID1	Inhibitor of DNA binding 1, HLH protein	0.236	0.419
NCSTN	Nicastrin	0.249	0.381
NCOR2	Nuclear receptor corepressor 2	0.299	0.177
DLL4	Delta like canonical Notch ligand 4	0.306	0.517
RBPJL	Recombination signal binding protein for immunoglobulin kappa J region like	0.339	0.396
SEL1L	SEL1L adaptor subunit of ERAD E3 ubiquitin ligase	0.341	0.679
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	0.342	0.180
TLE5	TLE family member 5, transcriptional modulator	0.386	0.461
VEGFD	Vascular endothelial growth factor D	0.390	0.234
HR	HR lysine demethylase and nuclear receptor corepressor	0.416	0.550
JAG2	Jagged canonical Notch ligand 2	0.420	0.707
KRT1	Keratin 1	0.428	0.255
CDKN1A	Cyclin dependent kinase inhibitor 1A	0.432	0.366
NEURL1	Neuralized E3 ubiquitin protein ligase 1	0.457	0.525

NOTCH2	Notch receptor 2	0.484	0.427
SHH	Sonic hedgehog signaling molecule	0.487	0.629
POFUT1	Protein O-fucosyltransferase 1	0.492	0.346
NOTCH2NL	Notch 2 N-terminal like A	0.509	0.440
CFLAR	CASP8 and FADD like apoptosis regulator	0.521	0.518
CTNNB1	Catenin beta 1	0.528	0.517
RFNG	RFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	0.551	0.682
HOXB4	Homeobox B4	0.561	0.640
NFKB1	Nuclear factor kappa B subunit 1	0.563	0.614
DTX1	Deltex E3 ubiquitin ligase 1	0.632	0.851
JAG1	Jagged canonical Notch ligand 1	0.645	0.412
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	0.658	0.800
HDAC1	Histone deacetylase 1	0.661	0.432
CCND1	Cyclin D1	0.676	0.833
GLI1	GLI family zinc finger 1	0.715	0.748
CCNE1	Cyclin E1	0.716	0.763
RUNX1	RUNX family transcription factor 1	0.774	0.750
DLL3	Delta like canonical Notch ligand 3	0.836	0.870
PSENEN	Presenilin enhancer, gamma-secretase subunit	1.004	0.997
SH2D1A	SH2 domain containing 1A	1.555	0.739
HES5	Hes family bHLH transcription factor 5	1.562	0.652
PSEN1	Presenilin 1	1.597	0.607
CD44	CD44 molecule (Indian blood group)	1.839	0.419
NOTCH3	Notch receptor 3	2.350	0.680

MMP7	Matrix metallopeptidase 7	2.430	0.704
LORICRIN	Loricrin cornified envelope precursor protein	2.950	0.455
NR4A2	Nuclear receptor subfamily 4 group A member 2	6.920	0.484

Differences in the ΔCt values for each gene between the cholesteatoma and control groups are analyzed by Welch's *t* test.


Fold change
10
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SUFU
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NCOR2
DLL4
RBPJL
SEL1L
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HR
JAG2
KRT1
CDKN1A
NEURL1
NOTCH2
SHH
POFUT1
NOTCH2NL
CFLAR
CTNNB1
RFNG
HOXB4
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LFNG
HDAC1
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GLI1
CCNE1
RUNX1
DLL3
PSENEN
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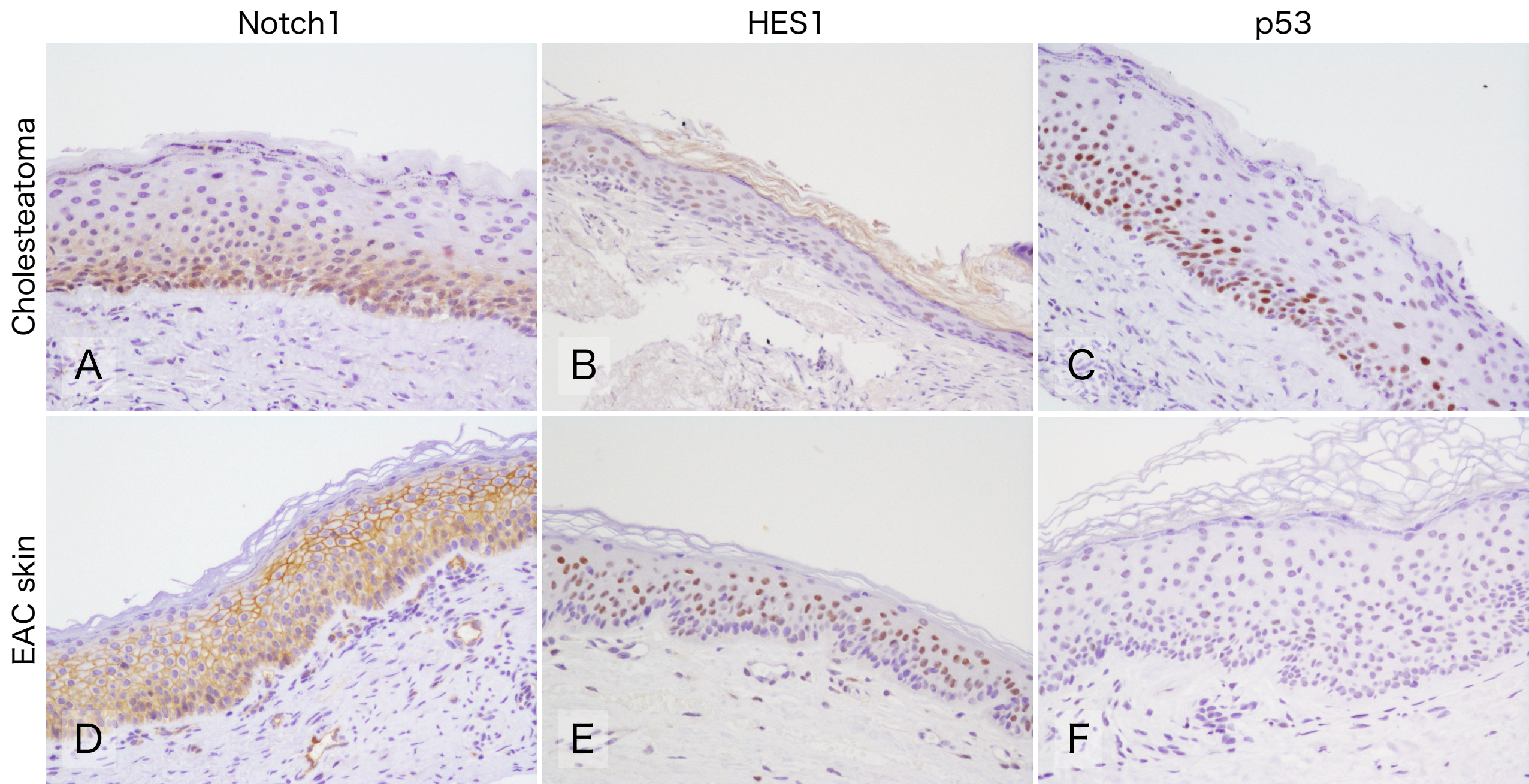
Notch1
HES1
p53
Cholesteatoma
EAC skin
A
B
C
D
E
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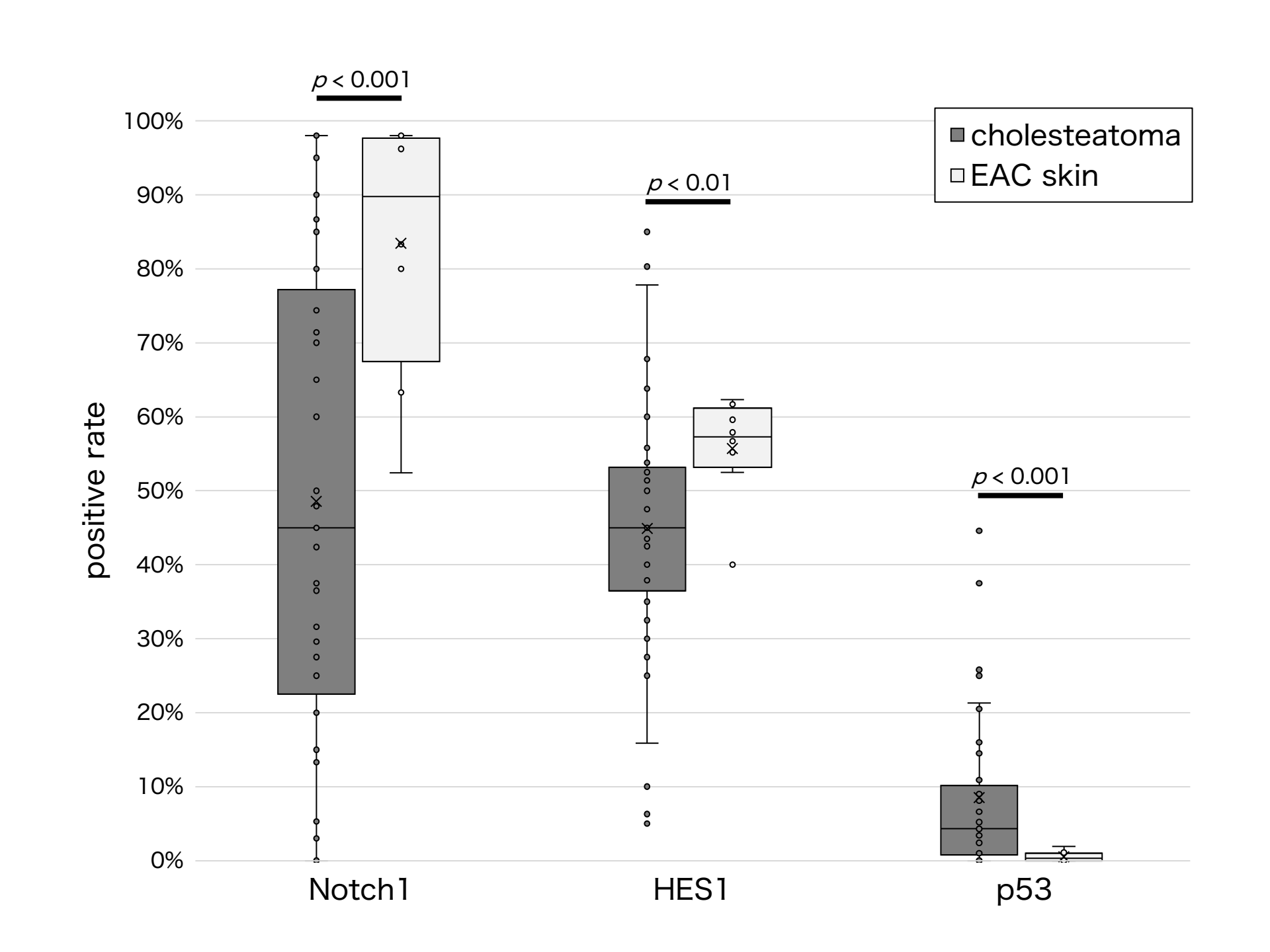
p < 0.001
p < 0.01
p < 0.001
cholesteatoma
EAC skin
positive rate
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Notch1
HES1
p53

Notch signaling pathway